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Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis-drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed, one the development and characterization of a homologous transformation system and two the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with goal of using this information in the identification and development of new antimalarial drugs.

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ANNUAL REPORT
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5. INTRODUCTION

Malaria - Drugs

Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis- drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem. Recognizing this emerging crisis, it is necessary to identify new strategies for the identification and development of new antimalarials. The goal of this work is the development of a framework for antimalarial drug development into the 21st century.

A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs based on an understanding of the parasite biology are key elements in this new strategy. Clearly the development of a new antimalarial will require both basic and applied research working in concert with one another.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed, one the development and characterization of a homologous transformation system and two the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with goal of using this information in the identification and development of new antimalarial drugs. This is a new strategy and it is being applied because of the crisis facing us in antimalarial drugs. The previous strategy, namely lead directed screening must be supplemented by new strategies or we will be faced with multiresistant *Plasmodium falciparum* and no drugs to treat it. There are two areas where they will be immediate application of this technology to pressing problems in malaria drug development.

Drug resistance and its implications for drug development

One of the major problems in infectious diseases today is that of drug resistance and multidrug resistance and this is a particularly acute problem for *Plasmodium falciparum* because there are only a few drugs available for safe and effective treatment and we are seeing evidence of cross-resistance and parasites with multiple resistant phenotypes. In parts of Southeast Asia and Brazil, there are reports *Plasmodium falciparum* resistant to all drugs which are currently registered for use in malaria treatment in the US and resistance to some

drugs which are still under evaluation and development. Thus, resistance is occurring faster than drugs are being developed.

This problem is not unique to malaria but is common in many infectious microorganisms. For example in HIV, there is rapidly emerging resistance to both inhibitors of reverse transcriptase and protease inhibitors. However, because we already have the tools of transfection and genetic manipulation, we are able to understand at the molecular level the mechanisms of this resistance in the HIV virus and investigators are able to develop strategies to use the available drugs in the most efficient way to prevent or delay the emergence of resistance and thus increase the useful life of the drug. For example, in the case of reverse transcriptase inhibitors in HIV infection, there are two different drugs currently in use, AZT and DDI. Each has a separate target in the protein which has been identified at the genetic level using methods of transfection and genetic engineering. With this knowledge, investigators are developing new lead compounds which are effective against viruses resistant to either AZT or DDI and importantly for today, have developed a strategy of alternating treatment with these drugs thus delaying the emergence of resistant organisms.

There are many similar examples in bacterial diseases as well, but the principle remains the same, the identification of the drug target and the analysis of resistance mechanisms is critical both to drug development and to effective use of drugs to delay the emergence of resistance. This is one of the applications of the new transfection/genetic engineering technology being developed in collaboration with WRAIR and Harvard. The first priority will be the investigation of drug resistance mechanisms with the goal of devising strategies either to prevent or overcome resistance.

In drug development today, one of the critical steps is the validation of the target. In other words, is the proposed target of the drug essential for the survival of the organisms and as a corollary to that, does the organism have alternate pathways to circumvent the inhibition of one enzyme. This is often the case for essential pathways and it is far better to know in the early stages of drug development whether this will be a problem. Malaria has severely lagged behind other diseases with regard to identification of drug targets and that is about to change based on this new transfection/genetic engineering technology.

6. BODY OF PROGRESS REPORT

Importance of drug resistance

Drug resistance has emerged as a major problem in the treatment of all microbial agents and in many cancer chemotherapy's. This has necessitated that continuous development of new chemotherapeutic agents both for treatment of infectious agents and for cancer chemotherapy. Often resistance develops through selection of a mutation in the target enzyme of the drug or in the overexpression of that enzyme. For example, resistance to antifolate drugs is frequently associated with mutations in the dihydrofolate reductase enzyme or in its overexpression. An alternative type of resistance, namely multidrug resistance has

emerged as a major problem in the treatment of many cancers and remains a major obstacle to the successful control of certain neoplasias with chemotherapy. This type of resistance is characterized by several unique features and the molecular basis for this resistance is under extensive investigation (Choi et al. 1991, Gros et al. 1986, Roninson et al. 1986, Raymond et al. 1990, Udea et al. 1987a, 1987b, Guild et al. 1988). In the case of multidrug resistance, resistance is observed to a number of structurally distinct drugs each with a different target. Selection of cells resistant to one drug results in the cross resistance to several structurally and functionally unique drugs. The genes associated with this resistance are the multidrug resistance mdr genes. The mdr gene encode membrane glycoproteins, the P-glycoprotein which mediate the efflux of drugs from the cell. Amplification of the mdr1 gene in resistant cells results in increased expression of the P-glycoprotein and thus increased efflux of drugs. Thus, the cells are resistant because drug is rapidly removed from the cell before significant toxicity occurs. Use of transfection of the mdr1 cDNA has demonstrated that overexpression of this gene is sufficient to confer the multidrug resistance phenotype. Drug resistance can be modulated by the use of several compounds including verapamil which appear to inhibit drug efflux. The current hypothesis is that verapamil and related compounds directly bind the P-glycoprotein molecule and block efflux and evidence for direct binding of radiolabelled verapamil to the P-glycoprotein molecule support this hypothesis.

In the case of malaria, the similarity in the pharmacological features of the chloroquine resistance in *P. falciparum*, namely the proposed efflux mechanism and the reversal of resistance by verapamil, desipramine and related compounds led to the proposal that a similar mechanism for drug resistance was operating in *P. falciparum* (Martin et al. 1987, Krogstad et al. 1992). Both our group under the support of this grant (Wilson et al. 1989, Foote et al. 1989) and David Kemp's group identified genes that had sequence and predicted structural similarity to the mdr genes and have completed the sequence, analyzed the expressed mRNA and protein. These genes and their encoded proteins are indeed related to the family genes in the ATP-Binding Cassette family and have the highest homology with the mdr genes from mouse and human. Thus, the hypothesis was proposed that these genes are involved in drug resistance in *P. falciparum*. Further evidence for this proposal was presented by the Foote et al. (1990) in identifying several polymorphism within the Pfmdr1 gene which appeared to be associated with chloroquine resistance in field isolates. This evidence was in contrast to the analysis by Wellems et al. in performing a genetic cross between a chloroquine resistant and chloroquine sensitive cloned parasite (Wellems et al. 1990, 1991). In the genetic analysis, both the pfmdr1 gene and its assorted polymorphism could be dissociated from chloroquine resistance. This was confirmed by a collaboration between our group and the NIH group in which we sequenced the relevant regions of the polymorphism from the resulting progeny (Wilson et al. submitted). Further evidence to refute the association of polymorphism in the pfmdr1 gene associated with chloroquine resistance was obtained by sequencing recent isolates of drug resistant *P. falciparum* (Wilson et al. submitted). We have completed this and have demonstrated in 12 new isolates of chloroquine resistant parasites, that the pfmdr1 gene sequence is identical to that predicted for the chloroquine sensitive phenotype, thus refuting the original Foote et al claim. Further RFLP analysis of the genetic cross by the Wellems group has determined linkage of the resistant phenotype to a small region of chromosome 7, a location distinct from the known location of either pfmdr1 or

pfmdr2. Thus, the conclusion from this work is that neither the pfmdr1 or pfmdr2 gene is linked to chloroquine resistance.

The mechanisms of chloroquine resistance remains unknown but progress has recently been reported on a putative target for chloroquine drug action. Slater et al. (1992) have reported an enzyme activity, heme synthetase which is hypothesized to be involved in the formation of hemozoan pigment and is a method for detoxification of the heme. This enzyme activity in cell extracts is inhibited by chloroquine and related quinones. Interestingly, the enzyme activity is equally sensitive to chloroquine whether derived from chloroquine sensitive or chloroquine resistant parasites. These results indicate progress towards identifying the primary target of chloroquine action and are consistent with the hypothesized importance of efflux of the chloroquine in drug resistance. The increased efflux phenotype remains associated with chloroquine resistance both in the genetic cross experiments and in new chloroquine resistant field isolates (Krogstadt et al. 1992, Wellems et al. 1990 and Watt et al. 1990). In addition reversal of chloroquine resistance with verapamil is observed in all chloroquine resistant strains tested. Thus, the pharmacology of this system remains consistent and has many similarities to the efflux mediated multidrug resistance in mammalian cells. However, the genetic evidence argues strongly that the identified pfmdr1 and pfmdr2 genes are not linked to the chloroquine resistance phenotype.

The role of pfmdr genes in other drug resistance mechanisms remains an open and important question. This is particularly the case for mefloquine resistance in Southeast Asia. In our original work, we demonstrated that in a laboratory selected mefloquine resistant cloned parasite, W2mef, the pfmdr1 gene was amplified when compared to the cloned parent parasite, W2. In subsequent work, Peel et al have demonstrated that under increased mefloquine selection pressure that the pfmdr1 gene is further amplified approximately 8-10 fold. We also demonstrated an increased expression of pfmdr1 mRNA in W2mef compared to W2. This work has now been expanded to include several field isolated of mefloquine resistant parasites and our data suggests that in mefloquine resistant parasites in Southeast Asia, an amplification of the pfmdr1 gene and an increased expression of mRNA is associated with this resistance (Volkman et al. 1992). Further, analysis of the mefloquine resistant strains from Southeast Asia demonstrates that they are cross-resistant, in vitro, to other unrelated drugs, similar to the cross-resistance observed in multidrug resistant mammalian cells (Wilson et al. submitted). Resistance to all drugs can be reversed by penfluoridol and other reversal compounds. Thus, it appears that mefloquine resistant *P. falciparum* has many of the characteristics in common with multidrug resistant mammalian cells, however, definitive proof of this relationship awaits functional analysis.

The protein encoded by the pfmdr1 gene has been identified both by our group and by Cowman and coworkers using antibody raised against fusion proteins. The P-glycoprotein molecule is 160,000 -170,000 MW and is found associated with membranes in fractionation studies. Cowman finds an association of the protein with the parasite food vacuole and proposes that it is involved with transport in and out of that vacuole. Further investigation of its localization throughout the parasite life cycle and in drug resistant verses drug sensitive parasites is necessary.

HYPOTHESIS

Drug resistance has emerged as a major problem in the treatment of all microbial agents and in many cancer chemotherapy's. Drug resistance has become particularly acute in malaria where resistance to chloroquine, the cheapest and most efficacious antimalaria has spread throughout the endemic parts of the world and resistance to other antimalarials is rapidly developing and spreading. The goal of this work is to understand the mechanism of drug resistance and to eventually use that information to develop new approaches to chemotherapy. The approach we propose is to develop a method for the functional analysis of genes in the malaria parasite. This methodology has proven invaluable in the analysis of drug resistance in other microbial systems, including our own recent work in leishmaniasis and particularly in multidrug resistance neoplastic cells. The initial aim of this work will be to test the function of genes implicated in drug resistance. This will be a multistep process in which we will first develop a method for the introduction and transient expression of foreign DNA into the parasite, we will then develop a method for the stable introduction of DNA using selectable markers and finally, we will test the role of the malaria mdr-like genes in drug resistance and in other parasite functions using methods of overexpression and gene knockout by homologous recombination. In parallel, we will continue development of the functional complementation system in yeast in which we are expressing the pfmdr1 gene. This system could prove extremely useful in testing drugs as potential resistance reversers.

TECHNICAL OBJECTIVES

1. Further development and characterization of the recently discovered transient transfection system in *Plasmodium gallinaceum* sexual stage parasites. This will include identification of putative promoter elements, transcriptional mapping, and testing of these vectors or modified vectors in other species.

2. Development of a system for stable transfection of the malaria parasite using a selectable marker.

3. Use of these transfection systems for the functional analysis of putative drug resistance genes in the malaria parasite.

4. Development of the heterologous yeast expression system for functional analysis of parasite genes and for the development of new testing systems for potential antimalarial drugs.

PROGRESS DURING THE REPORTING PERIOD

This report covers the first year of this work. Progress has been made in three of the technical objectives: 1) the further characterization of the transient transfection system in *Plasmodium gallinaceum* and *Plasmodium falciparum*; 2) strategy toward development of a

stable transfections system and 3) the use of the heterologous expression system in yeast to investigate the role of the pfmdr1 gene in drug resistance. A manuscript which describes the yeast work has been accepted for publication in the Proceedings of the National Academy of Science and is enclosed.

1. Further characterization of transient transfection systems

During this period we have completed the initial characterization of the transient transfection system in *Plasmodium gallinaceum* and have begun to transfer this technology into the *Plasmodium falciparum* system. The promoter region of the P. gallinaceum pgs28 gene has been defined by a series of nested deletion mutations and the minimal promoter region identified. We are now in the process of using site-specific mutagenesis to refine the precise location of the cis-acting elements in the pgs28 promoter regions. In parallel, we have now completed the initial characterization of the *Plasmodium falciparum* pfs25 gene and have developed a set of nested deletions for definition of this promoter region. Sequence analysis of these upstream regions is now in its final stages of completion and final confirmation of the sequence should be completed within the next quarter. It should be noted that the sequencing of this region has been particularly troublesome in that there are long stretches of sequence which are composed of a single base and this has created problems in resolving the sequence using both manual and automated sequencing methodologies.

We have begun to develop methods for the transfection of the asexual stages of the malaria parasite. Two approaches have been used. First, we have released the parasites from the infected red blood cells using the method of immune lysis and then introduced the plasmid DNA via electroporation. Secondly, we have attempted to directly electroporate infected red blood cells. This work initially used the *Plasmodium gallinaceum* asexual parasites. Using both methods, we have observed expression of the luciferase reporter gene consistent with successful transient transfection. The level of expression is much lower than that in the sexual stage parasites. We are attempting to modify the conditions of lectroporation and to develop transient transfection vectors which utilize promoters derived from genes expressed primarily in the asexual stage parasites.

In collaboration with Dr. Edwin Nuzum and his group at WRAIR, we have initiated work to develop a transient transfection system for *P. falciparum*. This work has involved three components, establishment of electroporation conditions using the hypoxanthine uptake assay to determine percent killing, development of plasmids for use in these assays and the testing of various drugs which might be used in conjunction with as selectable markers in a future stable transfections system. This work is ongoing with active communication including one working visit by Dr. Wirth and her student Wilfred Mbacham to WRAIR and several times weekly communications between the two groups.

2. Strategy toward the development of a stable transfection system

Our strategy for developing a stable transfection system is first to identify promoter and other gene controlling elements necessary for gene expression using the transient

transfection system and then replace the transient reporter gene with a selectable marker. Based on the drug testing assays done in collaboration with the WRAIR group, we have focused our efforts on the two selectable markers, Chloramphenicol Acetyle Transferase (CAT) and erythromycin resistance. In both cases, we have engineered plasmids which contain the selectable marker genes adjacent two promoter and 3 U RNA processing sites. These plasmids are now complete and we are in the process of confirming the structures through a combination of restriction mapping and DNA sequence analysis.

3. Heterologous expression system in yeast to investigate the role of the pfmdr1 gene in drug resistance

Excellent progress has been made in this section of the project. A manuscript accepted for publication is included as an appendix and the abstract of the manuscript is included below. We now are testing the effect of various antimalarial drugs and resistance reversers using this heterologous system.

Functional complementation of the ste6 gene of Saccharomyces cerevisiae with the pfmdr1 gene of *Plasmodium falciparum*

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The pfmdr1 gene has been associated with a drug resistant phenotype in *Plasmodium falciparum* and overexpression of pfmdr1 has been associated with mefloquine and halofantrine resistant parasites, but little is known about the functional role of pfmdr1 in this process. Here we demonstrate that the pfmdr1 gene expressed in a heterologous yeast system functions as a transport molecule and complements a mutation in ste6, a gene which encodes a mating pheromone a-factor export molecule. In addition, the pfmdr1 gene containing two mutations which are associated with naturally occurring chloroquine resistance, abolish this mating phenotype suggesting that these genetic polymorphisms alter this transport function. Our results support the functional role of pfmdr1 as a transport molecule in the mediation of drug resistance and provides an assay system to address the nature of this transport function.

Proc. Natl. Acad. Sci USA (in press)

7. CONCLUSIONS

We have made significant progress on three e of the four technical objectives. Ongoing work to develop a stable transfection system has the highest priority for the next year. The development of the heterologous test system in yeast should greatly facilitate our ability to analyze both antimalarial drugs and resistance reversers. This may provide the basis for a high throughput rapid screening procedure.

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